THE CULTIVATION OF ONE GENERATION OF BENIGN TERTIAN MALARIAL PARASITES (*PLASMODIUM VIVAX*) IN VITRO, BY BASS'S METHOD

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PLATE XIV

INTRODUCTION

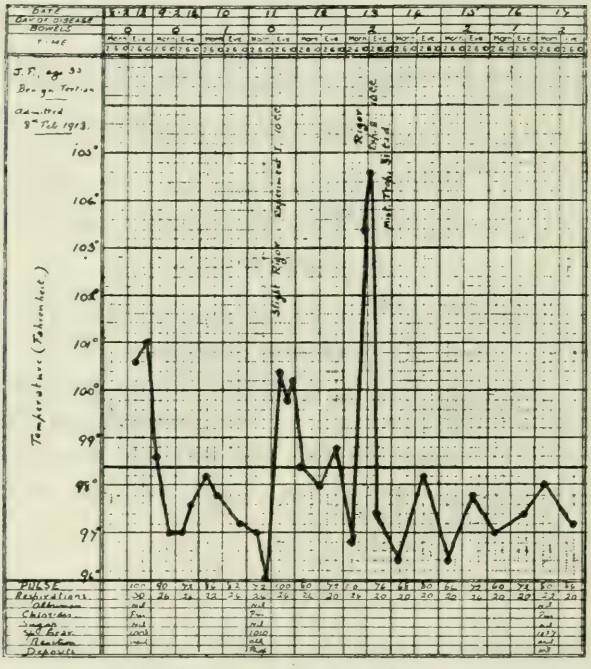
In this Journal (Vol. VI, No. 4, Dec. 1912) J. G. Thomson and S. W. McLellan described the cultivation of one generation of *Plasmodium falciparum*. We are now able to give the following details of the cultivation in vitro of one generation of *Plasmodium vivax*. It may be mentioned that this parasite was cultivated up to three-quarters growth by Dr. H. Carter and one of us (D. T.) in Panama in December last.

CASE

J. F., male, aged 35, was admitted to the Royal Southern Hospital, Liverpool, to Dr. Lloyd Roberts's clinic, in February, 1913.

The patient had resided for some time in the Island of Java, where he developed fever, with vomiting. On admission to hospital a history of four weeks' illness was given, and he complained of rigors, fever, aching pains in the limbs and back, and severe vomiting. Examination of his blood showed numerous young ring

parasites. The temperature on admission was 101° F. It dropped, without quinine treatment, and on the third day another rigor occurred, the temperature rising to 100·4° F. Ten c.c. of blood were taken, while the temperature was at its height, for cultivation of the parasites. Next day the fever had subsided, but on the following day another severe rigor occurred, the temperature rising to 104·6° F. Ten c.c. of blood were again taken from a vein, at the height of the fever, for purposes of cultivation. Quinine was then given in doses of ten grains thrice daily by the mouth, and no further attacks of fever resulted, the patient making a good recovery. The temperature chart is given below.



TECHNIQUE

As stated, 10 c.c. of blood were drawn, with aseptic precautions, from the median basilic vein on two occasions, at the time when the temperature was at its height, on the 11th February and again on the 13th February (see chart).

The blood on both occasions was transferred to a large sterile test tube and defibrinated; it was then distributed into several smaller test tubes and incubated at 39° C. The corpuscles settled gradually, leaving about one half-inch of clear serum above them.

The amount of dextrose solution added was slightly more than the amount recommended by Bass (1912).

EXAMINATION OF CULTURES

Experiment I. A blood smear, taken at the time of the withdrawal of the first 10 c.c. of blood on the 11th February, showed 85 per cent. of young ring parasites and 15 per cent. of three-quarter grown forms, also a few gametocytes. After nineteen hours' incubation there were only 3 per cent. of young rings, 17 per cent. were one-quarter grown, while 70 per cent. were one-half to three-quarters grown, and 10 per cent. showed signs of presegmentation. After twenty-three hours the majority of the parasites were three-quarters grown.

No further examination was made till the forty-fifth hour of incubation, and it was then found that 80 per cent. were small rings, and 20 per cent. were one-half to three-quarters grown. Sporulation had, therefore, occurred between the twenty-third and the forty-fifth hours of incubation.

Experiment II. Blood smears taken at the time of the second withdrawal of 10 c.c. of blood, on 13th February, showed 96 per cent. of young rings, the remaining 4 per cent. of the parasites being about one-half grown (Pl. XIV, figs. 1-3). After eight hours' incubation, 4 per cent. were young rings, 80 per cent. were one-quarter grown, and the remaining 16 per cent. were three-quarters to full-grown (figs. 4-10). After twenty hours, 65 per cent. were one-half to three-quarters grown, with only 2 per cent. of young rings, and a few sporulating forms (figs. 11-17). After twenty-nine hours, 89 per cent. were young rings (fig. 18), 8 per cent. were three-quarters grown, and again a few sporulating forms were found. It is, therefore, evident that in this experiment the maximum sporulation, which we again unfortunately missed, occurred between the twentieth and twenty-ninth hours of incubation.

Although the blood was drawn during the height of the temperature, that is, when the majority of the parasites would have

been expected to be very young, yet they attained their full development and sporulated in little more than half the time which would have been thought requisite for this development.

This, however, is in accord with the results obtained in the cultivation of *P. falciparum* by J. G. Thomson and McLellan (1912). They found that sporulation occurred after twenty-six hours of incubation. We do not think that the 96 per cent. of young rings shown at the time the blood was drawn could have been twenty hours old, so that it must be concluded that growth took place more rapidly in the tube than it would have done in the circulating blood of the patient.

MORPHOLOGY OF THE PARASITES IN CULTURES

The following remarks refer to Experiment II, and are illustrated by the accompanying plate (Pl. XIV). The morphology of the benign tertian parasite (*Plasmodium vivax*), as it occurs in the peripheral circulation, is well known. Unlike the malignant parasite, all stages may be found in the peripheral blood. In malignant tertian, as a rule, only ring forms are found, as the further development of the parasite takes place in the capillaries of the internal organs. In the benign tertian several stages of the development are usually found in a single smear of peripheral blood. Even the full-grown parasites of benign tertian, which, by the way, are much larger than the full-grown malignant *Plasmodia*, are not arrested in the fine capillaries of the internal organs, but are apparently able to circulate freely. A pure case of benign tertian malaria, in which the parasites are all in the same stage of development, is very rarely obtained, yet, as a rule, the majority of the *Plasmodia* are found to be of the same age. In the cultivation of *Plasmodium vivax*, therefore, if a full-grown parasite, or even a sporulating form, is found, it must not be concluded that development has taken place in the culture tube. In order to avoid being deceived by this, we have made careful differential counts in the smears taken at different periods of cultivation.

In our cultures the blood was drawn shortly after sporulation of most of the parasites, so that the majority were young rings. Figs. 1 and 2 show corpuscles with young rings, and already the corpuscles contain many Schüffner's dots, and are somewhat

enlarged, having a diameter of 9μ and 10μ respectively. In fig. 3 the corpuscle is 11·5μ in diameter, and contains a parasite rather more than one-quarter grown.

After eight hours' incubation the blood was again examined, and it was found that the parasites had increased in size in a very marked degree; the majority were now one-quarter grown, and many were three-quarters to full-grown. Fig. 5 shows a parasite about one-quarter grown. Figs. 7 and 8 show half-grown parasites with scattered pigment and the chromatin greatly increased in quantity. Figs. 9 and 10 represent full-grown parasites, and show the collection of pigment into a loose mass and marked increase of chromatin.

In twenty hours the majority of the parasites were half, three-quarters, or full-grown, and several were sporulating (figs. 11-17). Fig. 15 shows the parasite with chromatin divided into seven particles, or daughter portions. Figs. 16 and 17 show typical sporulation, with production of 14 or 15 merozoites. In culture it appears that the number of spores produced in benign tertian is less than that produced in the malignant form of the parasite (Thomson and McLellan, 1912). The maximum number of spores in *Plasmodium vivax* averages about sixteen, but in some cases a few more may be produced.

The culture was not again examined until the twenty-ninth hour, and it was found that the majority of the parasites were then young rings (fig. 18). There was, therefore, evidence that sporulation had occurred and the young merozoites produced had entered new corpuscles. No further asexual development took place.

DISCUSSION OF RESULTS

In the paper by J. G. Thomson and McLellan (1912) it was noted that the parasites, after an incubation of twelve hours, showed a definite increase in size, the pigment being collected together into a circular compact mass. In twenty-four hours the malignant tertian parasite was found to undergo segmentation. The maximum number of merozoites counted was thirty, and it is quite probable that thirty-two is the greatest number of merozoites produced by *Plasmodium falciparum* after complete segmentation. In their remarks, J. G. Thomson and McLellan pointed out the

great difference of opinion among various observers regarding the number of spores produced by the segmentation of the malignant tertian parasites, and reasons were given for this.

In some slides of smears of the spleen and bone marrow made during autopsies of several cases of comatose malaria (*P. falciparum*), sent by Dr. James from Panama, very numerous sporulating parasites were found. The number of spores in these preparations was counted by Sir Ronald Ross and one of us (D. T.). The following table gives the number of spores found in one hundred fully-developed malignant tertian parasites (*P. falciparum*) from a spleen smear of a case of untreated comatose malaria, Panama:—

13	per	cent.	contained	32	spores.
2	,,	,,	,,	31	,,
11	,,	,,	,,	30	,,
7	,,	,,	,,	29	,,
25	,,	,,	,,	28	,,
3	,,	,,	,,	27	,,
17	,,	,,	,,	26	,,
2	,,	,,	,,	25	,,
10	,,	,,	,,	24	,,
3	,,	,,	,,	23	,,
5	,,	,,	,,	22	,,
2	,,	,,	,,	20	,,
100	,,	,,	,, from 20 to 32 spores.		

These numbers lead us to conclude that the maximum number of spores, after complete segmentation, may be as high as thirty-two. This is entirely in agreement with the findings in culture by J. G. Thomson and McLellan, and is a higher number than has, up till recently, been given. Some observers have put the number as low as eight to ten (Stephens and Christophers, 1908).

Again, in a placenta smear from a case of comatose malaria, *P. falciparum*, Panama, sporulating parasites were found which did not fill the entire corpuscle. The average number of spores found in these was only eight. The patient, however, had received 60 grains of quinine during the twenty-four hours preceding death, so that the sporulation was either atypical due to the quinine, or else the parasites had not reached their maximum development. Dr. James, of Panama, also believes that those forms containing few spores are not completely developed, or are atypical sporulating forms due to the action of quinine.

It may now be stated with confidence that the malignant tertian parasite is capable of producing, under favourable conditions, a maximum of thirty-two spores, as proved both by culture and by examination of autopsy smears of cases of untreated comatose malaria. Last year Sir Ronald Ross taught his students that the maximum number of spores produced by the malignant tertian parasite was 2^5, while that of the benign tertian parasite was 2^4.

Another point of great importance, noted in the cultivation of *P. falciparum* by Thomson and McLellan (1912), was that there was a great tendency to clumping of the parasites, both before and after segmentation. This point is of great interest since we have found no such tendency in the case of cultures of benign tertian. This phenomenon explains why the malignant tertian parasites accumulate in the capillaries of the internal organs after attaining a certain size, only the corpuscles containing the young forms being able to circulate in the peripheral blood. It would also explain the blocking of the capillaries in the brain in the comatose form of the disease.

In our cultivation of benign tertian, as already stated, no such tendency to clumping of the parasites has been noted, and they always remain well distributed throughout the smears. This observation is in accordance with what we should expect, since in the benign tertian there is little tendency to blocking of the capillaries, with the resulting coma and other pernicious symptoms. The corpuscles containing all stages in the development of this parasite are able to circulate in the peripheral blood.

Another marked feature to be made out in cultivation is the distribution of the pigment. In the figures given by J. G. Thomson and McLellan for malignant tertian, it is clearly shown that the pigment collects into a circular compact mass before segmentation occurs, and remains so until the complete formation of merozoites. This has also been seen in smears from the internal organs. In the cultivation of *P. vivax* the pigment does not collect so early in the growth of the parasite, and at no time does it appear to form so dense or compact a mass as found in cultures of *P. falciparum*.

There was no evidence of haemolysis in the culture tubes.

POSSIBLE FORMATION OF GAMETOCYTES IN CULTURES

In a paper by one of us (D. Thomson, 1911), it was concluded that crescents develop from the youngest forms of the asexual parasite when a certain amount of immunity or resistance towards the latter had developed in the human blood, and that the time required for their development into an adult gametocyte was about ten days. Reverting now to a consideration of the cultural development of *P. vivax*, it was found that only one generation of asexual parasites developed in the culture tubes. After the first sporulation the parasites remained small, and did not pass through another asexual phase. On the 5th day, however, it was noticed in our cultures (*P. vivax*) that the parasites had changed much in appearance, since all of them had become heavily pigmented and compact (figs. 19-22), *even the very youngest forms contained pigment* (fig. 19). There were no amoeboid straggling forms, all were round and compact, and varied in size from very small circular pigmented bodies to forms as large as 4μ to 5μ in diameter (fig. 22). The pigment was scattered more or less evenly throughout, and the chromatin consisted of a single mass (fig. 21). The protoplasm had a greyish-blue colour, quite unlike that of the asexual phase. On the eighth day there was a very marked increase in the number of large forms, varying from 4μ to 6μ in diameter (figs. 23, 24). All were circular, compact, and very heavily pigmented. On the tenth day the cultures had dried up too much to permit of a further examination.

We are doubtful as to the true explanation of these bodies. They suggest gametocyte formation from their appearance, being circular, compact, and heavily pigmented, and there were many more large forms on the eighth day (figs. 23, 24) than on the fifth day, which would appear to indicate that these bodies had been growing in size. On the other hand, we must consider the possibility that these are degenerating parasites, though it is difficult to understand why degenerate parasites should develop such a large amount of pigment, or why they should become compact and show an apparent slow growth. We were unable to obtain any flagellation in these bodies, and the corpuscles containing them appeared to be shrunken.

SUMMARY

1. The benign tertian malarial parasite is capable of being cultivated up to the stage of sporulation, for at least one generation.

2. In our cultures the growth of the parasite from young rings to sporulating forms took place more rapidly than in the blood of the patient.

3. The cultures of benign tertian differed from those of malignant tertian in that there was no tendency to clumping of the parasites in the former, either before or during sporulation.

4. This difference appears to us to explain in a satisfactory manner why only young forms of malignant tertian are found in the peripheral blood, as the clumping tendency of the larger forms causes them to be arrested in the finer capillaries of the internal organs. It also explains the tendency to pernicious symptoms, such as coma, in malignant tertian malaria. All stages of the benign tertian parasite are found in the peripheral blood, and there are seldom pernicious symptoms, because there is no tendency to clumping.

5. Heavily pigmented, compact parasites were found in our cultures of benign tertian on the fifth day. On the eighth day these had grown larger, and their appearance suggested the development of gametocytes.

6. The malignant tertian parasite (*P. falciparum*) is capable of producing, in maximum segmentation, thirty-two spores. On the other hand, benign tertian (*P. vivax*) produces, as a rule, during maximum segmentation, sixteen spores; sometimes more may be produced, but the number is never thirty-two.

7. The pigment in *P. falciparum* collects into a definite, circular, and very compact mass early in the growth of the parasite. On the other hand, during the growth of *P. vivax* the pigment remains scattered in definite granules throughout the body of the parasite, till just before segmentation, when it collects into a loose mass of granules in the centre of the full-grown *Plasmodium*.

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EXPLANATION OF PLATE XIV

The figures were drawn with an Abbé camera lucida, using ocular 4 and a Leitz 1/12th-inch oil immersion objective, from Romanowsky stained preparations. The pigment is represented by coarser dots in the protoplasm of the organism. The magnification is 1,600 diameters, approximately.

Figs. 1 and 2 represent young plasmodia from the peripheral blood before incubation, showing the corpuscles just beginning to enlarge. Schüffner's dots are well seen; no pigmentation of the parasite is observed.

Fig. 3. Parasite is rather more than one-quarter grown. Corpuscle gradually increasing in size. Chromatin increasing in quantity. Schüffner's dots present. Drawn immediately before incubation.

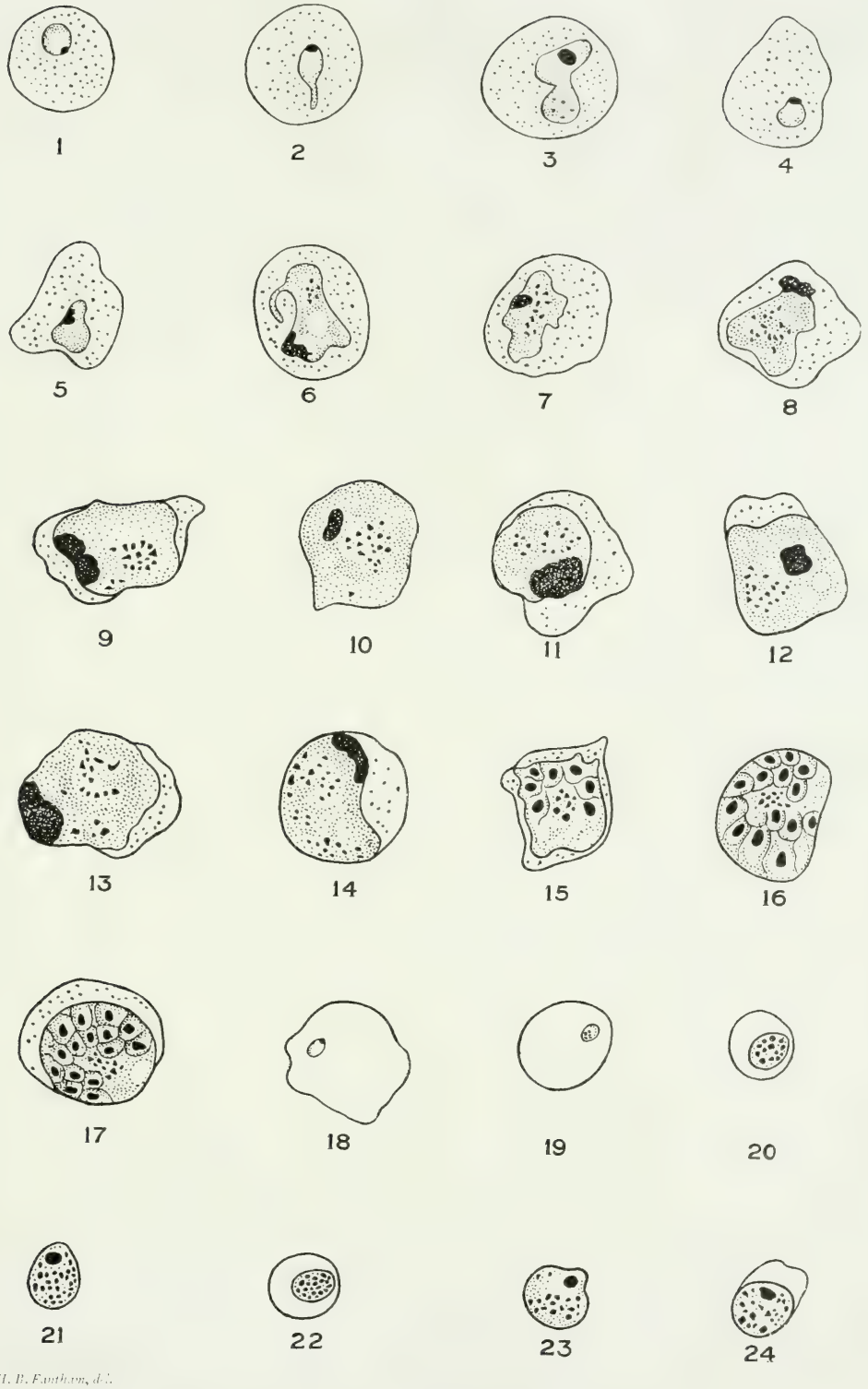
Figs. 4-10 represent parasites after eight hours' incubation, showing marked increase in size. Forms varying from small rings to the full-grown parasite. Figs. 7 and 8 represent half-grown forms. Figs. 9 and 10 represent practically full-grown forms. The parasites are now distinctly pigmented, and in figs. 9 and 10 the pigment is tending to collect in the centre of the parasite into a loose mass. Note also the great increase in the amount of chromatin.

Figs. 11-17 show parasites drawn after twenty hours' incubation. Figs. 15 to 17 show sporulating forms, the chromatin being divided into fifteen daughter portions in fig. 17.

Fig. 18. Young parasite after twenty-nine hours' incubation.

Figs. 19-22. Highly pigmented small parasites in culture of five days' incubation, suggesting the formation of young gametocytes.

Figs. 23-24 show parasites from eight days' culture. These are highly pigmented, and have increased in size. They are probably young gametocytes.



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CULTIVATION OF *PLASMODIUM VIVAX*

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